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Applicant(s): Sanders, et al.	
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Commissioner for Patents
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REQUEST FOR CORRECTED PUBLICATION

Sir:

The above-captioned patent application was published as US Publication No. 2006-0241289-A1 on October 26, 2006. Applicants respectfully request republication because several material errors were made in the publication. An unrelated Figure 5 was printed in the published document rather than the sequence listing depicted in the original Figure 5 included with the Preliminary Amendment filed with the application on May 27, 2005. In addition, none of the amendments to the specification made in the Preliminary Amendment were included in the publication.

Regarding the publication of amendments to the specification made in a Preliminary Amendment filed with an application, the Manual of Patent Examining Procedure § 1121 states that if the preliminary amendment is not in a format that is useable for publication, the Office will issue a notice requiring a substitute specification. Applicants never received such a notice and as such the USPTO did not follow its own procedure. A substitute specification is now

provided along with marked up copies of the relevant pages from the publication.

Finally, the publication retains the error in the title as addressed in the Request to Correct Filing Receipt applicants filed September 15, 2006. No fee is believed to be due with this request since these errors were made by the USPTO.

Respectfully,

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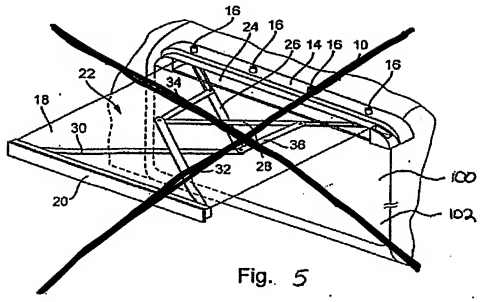

Fig. 5

Replace above figure with this figure.

QVQLVQSGAEVKKPGESLKISCRGSGYRFT[redacted]WVRQLPGKGLEWMG[redacted] 50

CDR I

[redacted]HVTVSADKSINTAYLQWSSLKASDTGMYYCAR[redacted] 100

CDR II

[redacted]WGQGTLVTVSSASTKGPSVFP 131

CDR III

constant region

SEQ ID NO: 5

Fig. 5

This application is a 371 national phase of PCT/GB2003/005171, filed November 28, 2003, and published in English as WO2004/050708 on June 17, 2004.

Binding Partners

~~ANTIBODY~~ FOR THE THYROTROPIN RECEPTOR AND USES THEREOF

Background of the Invention

[0001] The present invention is concerned with binding partners (such as monoclonal or recombinant antibodies) for the thyrotropin receptor (TSH receptor or TSHR) and uses thereof.

[0002] Thyrotropin or thyroid stimulating hormone (TSH) is a pituitary hormone which plays a key role in regulating the function of the thyroid. Its release is stimulated by the hormone TRH formed in the hypothalamus and TSH controls the formation and release of the important thyroid hormones thyroxine (T4) and tri-iodothyronine (T3). On the basis of a feedback mechanism, the thyroid hormone content of serum controls the release of TSH. The formation of T3 and T4 by thyroid cells is stimulated by TSH by a procedure in which the TSH released by the pituitary binds to the TSH receptor of the thyroid cell membrane.

[0003] In Graves' disease (a common autoimmune disorder) TSH receptor antibodies (TRAb) are formed and these autoantibodies bind to the TSH receptor in such a way as to mimic the actions of TSH, stimulating the thyroid gland to produce high levels of thyroid hormones. These autoantibodies are described as having stimulating activity. In some patients, autoantibodies bind to the TSH receptor but do not stimulate thyroid hormone production and are described as having blocking activity. (J Sanders, Y Oda, S-A Roberts, M Maruyama, J Furmaniak, B Rees Smith; "Understanding the thyrotrophin receptor function-structure relationship" Ballière's Clinical Endocrinology and Metabolism; Ed T F Davies 1997; 11(3): 451-479; pub Ballière Tindall, London).

[0004] Measurements of TSH receptor antibodies are important in the diagnosis and management of Graves' disease and other thyroid disorders. Currently three types of assay are used to measure TSH receptor antibodies:—

[0005] (a) competitive binding assays which measure the ability of TSH receptor antibodies to inhibit the binding of TSH to preparations of TSH receptor;

[0006] (b) bioassays which measure the ability of TSH receptor antibodies to stimulate cells expressing the TSH receptor in culture; and

[0007] (c) immunoprecipitation of TSH receptor preparations with TSH receptor antibodies.

[0008] Measurement of TSH receptor antibodies using such assays are described in references:—

[0009] J Sanders, Y Oda, S-A Roberts, M Maruyama, J Furmaniak, B Rees Smith; "Understanding the thyrotrophin receptor function-structure relationship" Ballière's Clinical Endocrinology and Metabolism; Ed T F Davies 1997; 11(3): 451-479; pub Ballière Tindall, London.

[0010] J Sanders, Y Oda, S Roberts, A Kiddie, T Richards, J Bolton, V McGrath, S Walters, D Jaskólski, J Furmaniak, B Rees Smith; "The interaction of TSH receptor autoantibodies with ^{125}I-labelled TSH receptor"; Journal of Clinical Endocrinology and Metabolism 1999; 84(10): 3797-3802.

[0011] It has been recognised for many years that human monoclonal antibodies to the TSH receptor derived from patients' lymphocytes would be valuable reagents for understanding the pathogenesis of Graves' disease and for developing new methods of measuring TSH receptor antibodies for example as replacements for TSH in competitive binding assays. Also, as the patient's serum TSH receptor antibodies are usually powerful thyroid stimulators (TSH agonists) stimulating human monoclonal TSH receptor antibodies would be valuable for in vivo applications when tissue containing the TSH receptor (eg thyroid tissue or thyroid cancer tissue) required stimulation. Furthermore, as some patient serum TSH receptor antibodies are powerful TSH antagonists (blocking antibodies) human monoclonal TSH receptor antibodies which are TSH antagonists would be valuable for in vivo applications when the activity of tissue containing the TSH receptor (eg thyroid tissue or thyroid cancer tissue) required inactivation or to be made unresponsive to TSH, TSH receptor antibodies or other stimulators.

[0012] It has also been recognised that one of the major advantages of human monoclonal TSH receptor antibodies over TSH in such in vitro and/or in vivo applications would be the relative ease with which antibodies can be manipulated. For example, manipulation of the TSH receptor binding region of the monoclonal antibodies so as to change their characteristics, such as affinity and biological characteristics including their degree of TSH agonist or antagonist activities. Also, monoclonal antibodies will have a much longer half life than TSH in vivo and this may have considerable advantages in certain in vivo applications. Furthermore, the half life of antibodies can be manipulated easily, for example antibody Fab fragments have a much shorter half life than intact IgG. These general properties of TSH receptor antibodies are described in the publications such as B Rees Smith, S M McLachlan, J Furmaniak; "Autoantibodies to the thyrotropin receptor"; Endocrine Reviews 1988; 9: 106-121; B Rees Smith, K J Dorrington, D S Munro; "The thyroid stimulating properties of long-acting thyroid stimulator γG-globulin subunits"; Biochimica et Biophysica Acta 1969; 192: 277-285; K J Dorrington, D S Munro; "The long acting thyroid stimulator"; Clinical Pharmacology and Therapeutics 1966; 7: 788-806.

[0013] A still further advantage of human monoclonal TSH receptor antibodies could be in their use to identify and provide new types of TSH receptor antibody binding sites. For example by the generation of antibodies to the regions of the human monoclonal TSH receptor antibodies which bind the TSH receptor. Some of the anti-idiotypic antibodies produced in this way could have potential as new ligands for assays of TSH receptor antibodies, TSH and related compounds. Also they may be effective agents in vivo for regulating the action of TSH receptor antibodies, TSH and related compounds.

[0014] Other methods of identifying and providing new types of antibody binding sites using monoclonal antibodies are well known. For example by antibody screening of phage-displayed random peptide libraries as described by JC Scott and GP Smith; "Searching for peptide ligands with an epitope library"; Science 1990; 249(4967): 386-390 and M A Myers, J M Davies, J C Tong, J Whisstock, M Scealy, I R MacKay, M J Rowley; "Conformational epitopes on the diabetes autoantigen GAD_{65} identified by peptide phage display and molecular modelling"; Journal of Immunology 2000; 165: 3830-3838. Antibody screening of non-peptide compounds and libraries of non-peptide compounds can also be carried out.

[0015] New types of TSH receptor antibody binding sites identified and provided using these procedures may also be

Insert attached paragraphs including "Summary of the Invention" and "Brief Description of Drawings" between paragraph 16 and paragraph 17.

useful as new ligands in assays for TSH receptor antibodies, TSH and related compounds. Furthermore they may be effective agents in vivo for regulating the action of TSH receptor antibodies, TSH and related compounds.

[0016] In view of the potential value of human monoclonal TSH receptor antibodies there have been considerable efforts over many years to produce such antibodies (see for example B Rees Smith, S M McLachlan, J Furmaniak; "Autoantibodies to the thyrotropin receptor"; Endocrine Reviews 1988; 9: 106-121. However, to date these efforts have been unsuccessful (see for example S M McLachlan, B Rapoport; "Monoclonal, human autoantibodies to the TSH receptor—The Holy Grail and why are we looking for it"; Journal of Clinical Endocrinology and Metabolism 1996; 81:3152-3154 and J H W van der Heijden, T W A de Bruin, K A F M Ghidenaas, J de Kruif, J P Banga, T Logtenberg; "Limitations of the semisynthetic library approach for obtaining human monoclonal autoantibodies to the thyrotropin receptor of Graves' disease"; Clinical and Experimental Immunology 1999; 118: 205-212).

[0017] It is an object of the present invention to provide a binding partner for the TSH receptor capable of interacting with the TSH receptor in a manner comparable to the interaction of TSH receptor autoantibodies with the TSH receptor, in particular it is an object of the present invention to provide human monoclonal antibodies to the TSH receptor exhibiting a comparable interaction therewith as seen with TSH receptor antibodies present in the sera of patients with hyperthyroid Graves' disease and also to provide recombinant preparations thereof. The considerable difficulties of producing human monoclonal TSH receptor antibodies have been overcome in the invention described herein. In particular the successful production of a human monoclonal TSH receptor antibody with the characteristics of the autoantibodies found in the sera of patients with hyperthyroid Graves' disease is described. The human TSH receptor monoclonal antibody we have produced (described herein as hMAb TSHR 1) binds to the TSH receptor with high affinity and in such a way that small amounts of the antibody inhibit labelled TSH binding to the TSH receptor and small amounts act as powerful thyroid stimulators. Fab fragments of the antibody and recombinant Fab preparations are similarly effective thyroid stimulators and inhibitors of labelled TSH binding as intact IgG. Monoclonal Fab and/or intact IgG can be labelled with ^{125}I or biotin and shown to bind to the TSH receptor. Such binding is inhibited by TSH receptor autoantibodies in patient sera.

[0018] There is provided by the present invention, therefore, a binding partner for the TSH receptor, which binding partner comprises, or is derived from, a human monoclonal or recombinant antibody, or one or more fragments thereof, reactive with the TSH receptor.

[0019] In particular, there is provided by the present invention a binding partner for the TSH receptor, which binding partner comprises, or is derived from, a human monoclonal antibody, or one or more fragments thereof, reactive with the TSH receptor.

[0020] In particular, there is provided by the present invention a binding partner for the TSH receptor, which binding partner comprises, or is derived from, a human recombinant antibody, or one or more fragments thereof, reactive with the TSH receptor.

[0021] In particular, there is provided by the present invention a human monoclonal antibody, or one or more fragments thereof, reactive with the TSH receptor.

[0022] In particular, there is provided by the present invention a human recombinant antibody, or one or more fragments thereof, reactive with the TSH receptor.

[0023] Particularly, the present invention provides one or more fragments of a human recombinant antibody reactive with the TSH receptor.

[0024] A binding partner according to the present invention, and in particular, a human monoclonal or recombinant antibody reactive with the TSH receptor according to the present invention can be further characterised by its ability to inhibit TSH binding to the TSH receptor, and/or its ability to stimulate the TSH receptor, both of which have been seen to be comparable to the respective inhibitory and stimulatory properties of TSH receptor autoantibodies present in sera obtained from patients with Graves' disease.

[0025] More particularly, a binding partner according to the present invention, and in particular a human monoclonal or recombinant antibody according to the present invention, can be characterised by an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 15 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or one or more fragments of such a monoclonal or recombinant antibody.

[0026] More particularly, a binding partner according to the present invention, and in particular a human monoclonal or recombinant antibody according to the present invention, can be further characterised by a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg, or one or more fragments of such a monoclonal or recombinant antibody.

[0027] In a preferred embodiment of the present invention, a binding partner according to the present invention, and in particular a human monoclonal or recombinant antibody according to the present invention, can be characterised by:

[0028] (i) an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 15 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg; and

[0029] (ii) a stimulatory activity with respect to cAMP production by cells expressing the TSH receptor, of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60

Insert the following between paragraph 16 and paragraph 17:

Summary of the Invention

The present provides a binding partner for TSH receptor. The binding partner comprises or is derived from

(a) a human monoclonal antibody reactive with the TSH receptor;
(b) a recombinant antibody reactive with the TSH receptor; or
(c) a fragment of a human monoclonal antibody or a recombinant antibody reactive with the TSH receptor.

The binding partner of the invention can be used in therapeutic and diagnostic applications, and for identification of epitope regions on TSH receptor.

Brief Description of the Drawings

Fig. 1 shows inhibition of TSH binding to TSH Receptor in the presence of a binding partner of the invention. The control IgG was a human monoclonal antobody to GAD_{65}.

Fig. 2 shows thyroid stimulating activity of hMAb TSHR1 IgG and Baf, porcine TSH (70 units/mg; pTSH) recombinant human TSH (6.7 units/mg; hTSH) and a control monoclonal antibody (MAb; human monoclonal autoantibody to thyroid peroxidase (2G4)). Basal = cAMP prpoduced in the presence of NaCl free Hanks Buffered Salt Solution only.

Fig. 3A shows the effect of lymphocyte donor serum on inhibition of TSH binding and on cAMP stimulation in THS receptor transfected CHO cells. In the case of the binding i nhibition assay the serum was diluted in a pool of healthy donor sera. For the stimulation assay, the serum was diluted in NaCl free Hanks Buffered Salt Solution. Healthy blood donor sera (n=3) gave responses ranging from 1.1 to 1.3 X basal.

Fig. 3B shows a comparison of an ELISA for TSHR autoantibodies according to thepresent invention with earlier assays: an ELISA on TSH-biotin described in Bolton et al., Clinical Chemistry

(1999) 45: 2285-2287 and the original RIA described by Southgate et al. in Clinical Endocrinology (1984) 20: 539-543.

Fig. 3C shows a comparison of an ELISA for TSHR autoantibodies according to the present invention and an ELISA based on TSH biotin as described in Bolton et al., Clinical Chemistry (1999) 45: 2285-2287. Sera from 72 patients with Graves disease were compared. y=1.1154x - 13.032 , r=0.99.

Fig. 4 shows the nucleotide sequence of hMAb TSHR1 Heavy chain V, D and J region with the primer, CDR and constant region sites labeled (Seq ID No: 14).

Fig. 5 shows the amino acid sequence of hMAb TSHR1 Heavy chain V, D and J region with the CDR and constant regions labeled (Seq ID No: 5).

Fig. 6 shows the nucleotide sequence of hMAb TSHR1 Light chain with the primer and CDR sites labeled (Seq ID No: 14).

Fig. 7 shows the amino acid sequence of hMAb TSHR1 Light chain with the CDR sites labeled (Seq ID No: 6).

Fig. 8 shows effects of 2 patients sera (T1 and T2 with TSH antagonist activity) on stimulation of cyclic AMP production by pTSH (0.5 ng/ml) and hMAb TSHR1 IgG (10 ng/ml) and Fab (5 ng/ml) in CHO cells transfected with the TSHR.

Fig. 9 shows the nucleotide sequence of 9D33 Heavy chain with primer, CDR and constant region sites marked. (Seq. ID No. 33).

Fig. 10 shows the amino acid sequence of 9D33 Heavy chain with primer, CDR and constant region sites marked. (Seq. ID No. 23).

Fig.11 shows the nucleotide sequence of 9D33 light chain with primer, CDR and constant region sites marked. (Seq. ID No. 38).

Fig. 12 shows the amino acid sequence of 9D33 light chain with primer, CDR and constant region sites marked. (Seq. ID No. 28).

Detailed Description of the Invention

[0096] For 9D33:

Amino Acid Sequences	
SEQ ID NO. 19	V_H
SEQ ID NO. 20	V_H CDRI
SEQ ID NO. 21	V_H CDRII
SEQ ID NO. 22	V_H CDRIII
SEQ ID NO. 23	Heavy chain variable and adjacent constant region
SEQ ID NO. 24	V_L
SEQ ID NO. 25	V_L CDRI
SEQ ID NO. 26	V_L CDRII
SEQ ID NO. 27	V_L CDRIII
SEQ ID NO. 28	Light chain variable and adjacent constant region

[0097]

Nucleotide Sequences	
SEQ ID NO. 29	V_H
SEQ ID NO. 30	V_H CDRI
SEQ ID NO. 31	V_H CDRII
SEQ ID NO. 32	V_H CDRIII
SEQ ID NO. 33	Heavy chain variable and adjacent constant region
SEQ ID NO. 34	V_L
SEQ ID NO. 35	V_L CDRI
SEQ ID NO. 36	V_L CDRII
SEQ ID NO. 37	V_L CDRIII
SEQ ID NO. 38	Light chain variable and adjacent constant region

[0098] The above sequences for hMab TSHR1 can also be seen by reference to **FIGS. 4, 5, 6 and 7**, wherein:

[0099] **FIG. 4** shows the hMab TSHR1 heavy chain nucleotide sequence, along with the adjacent constant region, ~~with~~

[0100] ~~FIG. 4a giving the nucleotide sequence per se;~~

[0101] ~~FIG. 4b giving the nucleotide sequence~~ annotated with the PCR primer, CDRI, CDRII, CDRIII and constant regions;

[0102] **FIG. 5** shows the hMab TSHR1 heavy chain amino acid sequence, along with the adjacent constant region, ~~with~~

[0103] ~~FIG. 5a giving the amino acid sequence per se;~~

[0104] ~~FIG. 5b giving the amino acid sequence~~ annotated with the CDRI, CDRII, CDRIII and constant regions;

[0105] **FIG. 6** shows the hMab TSHR1 light chain nucleotide sequence, ~~with~~

[0106] ~~FIG. 6a giving the nucleotide sequence per se;~~

[0107] ~~FIG. 6b giving the nucleotide sequence~~ annotated with the PCR primer, CDRI, CDRII and CDRIII regions; and

[0108] **FIG. 7** shows the hMab TSHR1 light chain amino acid sequence, ~~with~~

[0109] ~~FIG. 7a giving the amino acid sequence per se;~~

[0110] ~~FIG. 7b giving the amino acid sequence~~ annotated with the CDRI, CDRII and CDRIII regions.

[0111] It will be appreciated from the above that for the V_H chain of hMab TSHR1 the nucleotide sequences of the CDRI, CDRII and CDRIII regions as shown in **FIG. 4** correspond to the V_HCDRI, V_HCDRII and V_HCDRIII sequences shown in SEQ ID NO.s 11, 12 and 13 respectively, and that the amino acid sequences of the CDRI, CDRII and CDRIII regions as shown in **FIG. 5** correspond to the V_HCDRI, V_HCDRII and V_HCDRIII sequences shown in SEQ ID NO.s 2, 3 and 4 respectively. It will also be appreciated from the above that for the V_L chain of hMab TSHR1 the nucleotide sequences of the CDRI, CDRII and CDRIII regions as shown in **FIG. 6** correspond to the V_LCDRI, V_LCDRII and V_LCDRIII sequences shown in SEQ ID NO.s 16, 17 and 18 respectively, and that the amino acid sequences of the CDRI, CDRII and CDRIII regions as shown in **FIG. 7** correspond to the V_LCDRI, V_LCDRII and V_LCDRIII sequences shown in SEQ ID NO.s 7, 8 and 9 respectively.

[0112] Analysis of the crystal structure of hMab TSHR1 Fab (determined by techniques known in the art) enabled refinement of the HC and LC nucleotide sequences determined using PCR primers which are degenerate. In particular, a HC sequencing artefact for nucleotides 115-120 was identified. Sequencing indicated cacgtg (transcribed to amino acids His Val), whereas the crystal structure more reliably indicated amino acids Gln Leu (corresponding bases being cagctg), with the refined sequences being shown in the accompanying Figures and Sequence listings. Crystal structure analysis also enabled refinement of the HC and LC derived amino acid sequences particularly in the degenerate PCR primer region. In the case of the LC an 2 was found to be Pro by RT-PCR but was Thr from the crystal structure. In the case of the HC an 2 was found to be Met by RT-PCR but was Val from the crystal structure. Again, these refined sequences are shown in the accompanying Figures and Sequence listings.

[0113] The above sequences for 9D33 can also be seen by reference to **FIGS. 9, 10, 11 and 12**, wherein:

[0114] **FIG. 9** shows the 9D33 heavy chain nucleotide sequence, along with the adjacent constant region, ~~with~~

[0115] ~~FIG. 9a giving the nucleotide sequence per se;~~

[0116] ~~FIG. 9b giving the nucleotide sequence~~ annotated with the PCR primer, CDRI, CDRII, CDRIII and constant regions;

[0117] **FIG. 10** shows the 9D33 heavy chain amino acid sequence, along with the adjacent constant region, ~~with~~

[0118] ~~FIG. 10a giving the amino acid sequence per se;~~

[0119] ~~FIG. 10b giving the amino acid sequence~~ annotated with the PCR primer, CDRI, CDRII, CDRIII and constant regions;

[0120] **FIG. 11** shows the 9D33 light chain nucleotide sequence, ~~with~~

[0121] ~~FIG. 11a giving the nucleotide sequence per se;~~

[0122] ~~FIG. 11b giving the nucleotide sequence~~ annotated with the PCR primer, CDRI, CDRI, CDRII and constant regions;

[0123] **FIG. 12** shows the 9D33 light chain amino acid sequence, ~~with~~

[0124] ~~FIG. 12a giving the amino acid sequence per se;~~

[0125] ~~FIG. 12b giving the amino acid sequence~~ annotated with the PCR primer, CDR1, CDR11, CDR111 and constant regions.

[0126] It will be appreciated from the above that for the V_L chain of 9D33 the nucleotide sequences of the CDR1, CDR11 and CDR111 regions as shown in **FIG. 9** correspond to the V_HCDR1, V_HCDR11 and $V_HCDR111$ sequences shown in SEQ ID NO.s 30, 31 and 32 respectively, and that the amino acid sequences of the CDR1, CDR11 and CDR111 regions as shown in **FIG. 10** correspond to the V_HCDR1, V_HCDR11 and $V_HCDR111$ sequences shown in SEQ ID NO.s 20, 21 and 22 respectively. It will also be appreciated from the above that for the V_L chain of 9D33 the nucleotide sequences of the CDR1, CDR11 and CDR111 regions as shown in **FIG. 11** correspond to the V_LCDR1, V_LCDR11 and $V_LCDR111$ sequences shown in SEQ ID NO.s 35, 36 and 37 respectively, and that the amino acid sequences of the CDR1, CDR11 and CDR111 regions as shown in **FIG. 12** correspond to the V_LCDR1, V_LCDR11 and $V_LCDR111$ sequences shown in SEQ ID NO.s 25, 26 and 27 respectively.

[0127] The present invention also provides a process of providing a human monoclonal antibody to the TSH receptor substantially as hereinbefore described, which process comprises:

[0128] (i) providing a source of lymphocytes from a subject, which subject has TSH receptor antibody activity of greater than about 0.04 units of NIBSC 90/672 per mL of serum with respect to inhibition of TSH binding to the TSH receptor;

[0129] (ii) isolating lymphocytes from said lymphocyte source of (i);

[0130] (iii) immortalising the isolated lymphocytes; and

[0131] (iv) cloning the immortalised lymphocytes so as to produce an immortalised colony secreting a human monoclonal antibody to the TSH receptor substantially as hereinbefore described.

[0132] Alternatively, a process of providing a human monoclonal antibody to the TSH receptor substantially as hereinbefore described can be defined as a process which comprises:

[0133] (i) providing a source of lymphocytes from a subject, which subject has TSH receptor antibody activity of greater than about 0.1 units of NIBSC 90/672 per mL of serum with respect to stimulatory activity of cAMP production by cells expressing the TSH receptor;

[0134] (ii) isolating lymphocytes from said lymphocyte source of (i);

[0135] (iii) immortalising the isolated lymphocytes; and

[0136] (iv) cloning the immortalised lymphocytes so as to produce an immortalised colony secreting a human monoclonal antibody to the TSH receptor substantially as hereinbefore described.

[0137] Preferably a process according to the present invention comprises isolating lymphocytes from peripheral blood, thyroid tissue, spleen tissue, lymph nodes or bone marrow, most typically from peripheral blood. Typically, the source of lymphocytes for use in a method according to the present invention can be further characterised as being obtained from a subject having serum TSH receptor antibody levels of greater than about 0.1 units of NIBSC 90/672 per mL with respect to inhibition of TSH binding to the TSH receptor, or more typically greater than about 0.2 units of NIBSC 90/672 per mL with respect to inhibition of TSH binding to the TSH receptor, or more typically greater than about 0.3 units of NIBSC 90/672 per mL with respect to inhibition of TSH binding to the TSH receptor and preferably being in the range of about 0.3 to 0.5 units of NIBSC 90/672 per mL or greater with respect to inhibition of TSH binding to the TSH receptor. Alternatively, or additionally, the source of lymphocytes for use in a method according to the present invention can typically be further characterised as being obtained from a subject having serum TSH receptor antibody levels of greater than about 0.2 units of NIBSC 90/672 per mL with respect to stimulatory activity of cAMP production by cells expressing the TSH receptor, or more typically greater than about 0.5 units of NIBSC 90/672 per mL with respect to stimulatory activity of cAMP production by cells expressing the TSH receptor and preferably being in the range of about 0.5 to 1.0 units of NIBSC 90/672 per mL or greater with respect to stimulatory activity of cAMP production by cells expressing the TSH receptor. It will be appreciated from the above that the immune response to the TSH receptor of a subject from which lymphocytes are isolated should preferably be in a highly active phase.

[0138] Preferably a process according to the present invention comprises infecting the isolated lymphocytes with Epstein Barr virus, and suitably the thus immortalised lymphocytes are fused with a mouse/human cell line. Suitably a process according to the present invention further comprises screening the resulting clones for TSH receptor antibodies, for example by inhibition of 125I TSH binding to the TSH receptor in an assay system which has a sensitivity of at least about 1 unit/L of NIBSC 90/672.

[0139] The present invention further provides a process of preparing a human recombinant antibody, or one or more fragments thereof, to the TSH receptor, which process comprises cloning and expression of a human monoclonal antibody to the TSH receptor as provided by the present invention by a process substantially as hereinbefore described, or one or more fragments derived therefrom.

[0140] The present invention further provides a human monoclonal or recombinant antibody to the TSH receptor obtained by a process substantially as described above. Preferably such an obtained human monoclonal or recombinant antibody to the TSH receptor according to the present invention, can be characterised by an inhibitory activity with respect to TSH binding to the TSH receptor, of at least about 15 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, or more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or one or more fragments of such a human monoclonal or recombinant antibody.

least about 30 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 60 units of International Standard NIBSC 90/672 per mg, more preferably of at least about 120 units of International Standard NIBSC 90/672 per mg, or even more preferably of at least about 240 units of International Standard NIBSC 90/672 per mg;

or one or more fragments thereof.

[0154] A binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention may have diagnostic and therapeutic applications.

[0155] Accordingly, a binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention can be employed in screening methods for detecting autoantibodies to the TSH receptor in patient sera and also in diagnostic methods. In this way, a binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention can be employed in place of, or in addition to, competitors hitherto described for use in screening methods for detecting autoantibodies to the TSH receptor and also in diagnostic methods. Similarly, a binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention can be employed in place of, or in addition to, competitors hitherto described for use in kits for use in detecting autoantibodies to the TSH receptor.

[0156] The present invention also provides, therefore, a method of screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to a TSH receptor, said method comprising:

[0157] (a) providing said sample of body fluid from said subject;

[0158] (b) providing one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention and a second molecule of said binding pair comprises a binding region with which said binding partner or further binding partner interacts;

[0159] (c) contacting said sample with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) said binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody); and

[0160] (d) monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

[0161] A method according to the present invention for the detection of autoantibodies as described above is particularly advantageous in terms of the level of sensitivity that can be achieved by use thereof. This can be further illustrated by reference to the Examples and Figures, where **FIG.** 38 shows a graphical representation of a comparison between an assay for TSHR autoantibodies based on hMAb TSHR1-biotin and earlier assays. The sensitivity of the assay based on hMAb TSHR1-biotin is clearly superior according to concentration of the international standard NIBSC 90/672 detectable. This was confirmed in a study of sera from 72 patients with Graves' disease shown in **FIG.** 38.

[0162] There is further provided by the present invention, therefore, a method of screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a subject suspected of suffering from, susceptible to, having or recovering from autoimmune disease associated with an immune reaction to the TSH receptor, said method comprising:

[0163] (a) providing said sample of body fluid from said subject;

[0164] (b) providing one or more pairs of binding molecules, wherein a first molecule of said binding pair comprises a binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention and a second molecule of said binding pair comprises a binding region with which said binding partner or further binding partner interacts, wherein the interaction of said binding molecules is such that an autoantibody titer in said sample essentially corresponding to 0.4U/L of International Standard NIBSC 90/672 is detectable;

[0165] (c) contacting said sample with said one or more pairs of binding molecules so as to permit said second molecule of said binding pair to interact with either (i) autoantibodies to the TSH receptor present in said sample, or (ii) said binding partner or further binding partner for the TSH receptor (typically a human monoclonal or recombinant antibody) according to the present invention; and

[0166] (d) monitoring the interaction of said second molecule of said binding pair with said autoantibodies present in said sample, thereby providing an indication of the presence of said autoantibodies to the TSH receptor in said sample.

[0167] The above sensitivity can also be achieved in an assay method or kit according to the present invention by the use of a human or non-human polyclonal antibody to the TSH receptor, TSH or one or more variants, analogs, derivatives or fragments thereof, or a binding partner for the TSH receptor which has an affinity for the TSH receptor of 10^{10} $molar^{-1}$ or greater, which generally exhibit a sufficient affinity for the TSH receptor so that a method or kit of the defined sensitivity is provided. The preparation of such polyclonal antibodies, TSH or one or more variants, analogs, derivatives or fragments thereof, is well known in the art. For example, superactive analogs of TSH are described in Nature, Biotechnology, Volume 14, October 1995, pages 1257-1263, although this article does not disclose the use of such superactive TSH in a method or kit as is now provided by the present invention.

[0168] There is further provided by the present invention, therefore, a method of screening for autoantibodies to the TSH receptor in a sample of body fluid obtained from a

bated for 1 hour at room temperature in plastic tubes coated with Fab specific goat anti human IgG (from Sigma Aldrich, Poole, BH12 4QH, UK). The tubes were then washed with assay buffer (2x1 mL) and 100 μL of ^{125}I-labelled hMAb TSHR1 Fab (30,000 cpm) added followed by incubation at room temperature. After 1 hour, the tubes were washed again (2×1 mL) and counted for ^{125}I. The counts bound were plotted against concentration of Fab (hybridoma produced hMAb TSHR1 Fab) in the calibrators (5-500 ng/mL) and the concentration of recombinant Fab in the various test materials read off this calibration curve. The detection limit for this assay was 5-10 ng/mL of Fab.

Cloning and Expression of Recombinant 4B4 (a Human MAb to Glutamic Acid Decarboxylase or GAD) and Recombinant Hybrid Fabs (Mixed HC and LC of hMAb TSHR1 and 4B4)

[0295] Recombinant 4B4 Fab (4B4 is described in detail by N Hayakawa, LDKE Premawardhana, M Powell, M Masuda, C Arnold, J Sanders, M Evans, S Chen, J C Jaume, S Baekkeskov, B Rees Smith, J Furmaniak. Isolation and characterization of human monoclonal antibodies to glutamic acid decarboxylase. Autoimmunity 2002 volume 35 pp 343-355) and recombinant hybrid Fabs were produced by cloning the respective HC and LC into Immunozap H/L vector and expressed in E1B2151 cells as described for recombinant hMAb TSHR1 Fab. The culture supernatants and periplasmic fraction were assayed for their ability to (a) inhibit TSH binding to the TSHR (b) to stimulate cyclic AMP production in CHO cells expressing the TSHR and (c) for total recombinant Fab concentration as described above. In addition, GAD Ab activity was assessed as described below.

Measurement of Recombinant GAD Ab Fab Activity in Culture Supernatants and Periplasmic Fractions

[0296] An assay based on the ability of GAD Ab Fab preparation to inhibit the binding of ^{125}I-labelled GAD (from RSR Ltd, Cardiff, CF23 SHE, UK) to the human monoclonal antibody to GAD (4B4) was used. In the assay, test samples diluted in GAD Ab assay buffer (150 mmol/L NaCl, 50 mmol/L Tris-HCl pH 8.0, 1% v/v Tween 20, 1 g/L bovine serum albumin and 0.5 g/L NaN_3) were incubated (50 μL in duplicate with ^{125}I-labelled GAD (30,000 cpm in 50 μL of GAD Ab assay buffer) for 1 hour at room temperature. Then, 50 μL of 4B4 IgG (0.1 μg/mL in GAD Ab assay buffer) was added and incubation continued for 24 hours at room temperature. Solid phase protein A (50 μL in GAD Ab assay buffer; from RSR Ltd) was then added to precipitate complexes of 4B4 IgG-125I-labelled GAD (protein A does not react with complexes of Fab and ^{125}I-labelled GAD). After allowing the reaction with protein A to proceed for 1 hour at room temperature, the precipitates were pelleted by centrifugation (1500 g for 30 minutes at 4° C.), washed with 1 mL of GAD Ab assay buffer and counted for 1251. ^{125}I-labelled GAD binding in the absence of 4B4 IgG was 4-5% of the total cpm added.

Production of Anti-Idiotypic Antibodies to hMAb TSHR1

[0297] 6-8 week old BALB/c mice were immunised intraperitoneally with 50 μg hMAb TSHR1 Fab in complete Freunds adjuvant followed by a second injection with 50 μg hMAb TSHR1 Fab in incomplete Freunds adjuvant after 25 days and a further injection of 50 μg hMAb TSHR1 Fab 4 days before removal of the spleen. The spleen cells from antibody positive mice (see below) were fused with a mouse myeloma cell line and monoclonal antibodies isolated as above for TSHR MAbs. The levels of anti-idiotypic antibody in the mouse sera and cell culture wells were measured by inhibition of ^{125}I-hMAb TSHR1 Fab binding to TSHR coated tubes. In particular, duplicate 60 μL aliquots of test sample (diluted in assay buffer: 50 mmol/L NaCl, 10 mmol/L Tris-HCl pH 7.8, 0.1% Triton X-100) were incubated with 60 μL of ^{125}I-hMAb TSHR1 Fab (30 000 cpm diluted in assay buffer) for 1 hour at room temperature. 100 μL of the mixture was transferred to duplicate TSHR coated tubes (RSR Ltd) with 20 μL start buffer (see above) and incubation continued for a further two hours at room temperature with shaking. The tubes were then washed with 2×1 mL of assay buffer and counted for ^{125}I. The presence of anti-idiotypic antibodies reactive with hMAb TSHR1 was evident from the ability of test samples to inhibit the binding of labelled hMAb TSHR1 Fab to the TSHR coated tubes.

Results

[0298] Lymphocytes (30×10^6) obtained from 20 mL of patient's blood were plated out at 1×10^6 per well on a 48 well plate with 200 μL of EBV supernatant on feeder layers of mouse macrophages. On day 11 post EBV infection the supernatants were monitored for inhibition of ^{125}I-TSH binding. One well was found to be positive for inhibition of binding, the levels of inhibition increasing to greater than 90% inhibition by day 16 and stayed at that level until day 24, after which time they decreased. The cultures were expanded and fused with K6H6/B5 cells on day 21, 23, 26 and 27 post EBV infection; in total 7 fusion experiments were carried out. Each fusion was plated across 3×96 well plates (ie 21 plates in total) and one well, stably producing antibodies with ^{125}I-TSH binding inhibiting activity was obtained. This was followed by 3 rounds of re-cloning to yield a single clone producing a human monoclonal antibody which inhibited labelled TSH binding to the TSH receptor. This human monoclonal TSH receptor autoantibody was designated hMAb TSHR1 and was of subclass IgG1 with a lambda light chain.

[0299] The ability of different concentrations of hMAb TSHR1 IgG and Fab to inhibit labelled TSH binding to the TSH receptor is shown in FIG. 1. As can be seen in FIG. 1 as little as 1 ng/mL of these preparations inhibited TSH binding with more than 90% inhibition being obtained with 1000 ng/mL. TSMAb TSHR1 IgG and Fab also stimulated cyclic AMP production in CHO cells transfected with the TSH receptor as shown in FIG. 2. As little as 1 ng/mL of hMAb TSHR1 IgG or Fab caused strong stimulation of cyclic AMP. Similar levels of stimulation were observed with 0.1 ng/mL porcine TSH and 10 ng/mL of human TSH. Comparison of the ability of the serum from the original lymphocyte donor (taken at the same time as the blood sample for lymphocyte isolation) to inhibit labelled TSH binding to the TSH receptor and to stimulate cyclic AMP production in TSH receptor transfected CHO cells is shown in FIG. 3. Inhibition of TSH binding could be detected with serum diluted 500× whereas stimulation of cyclic AMP could be detected with serum diluted 5000×.

3A

[0300] ^{125}I-labelled hMAb TSHR1 IgG bound to TSH receptor coated tubes and Scatchard analysis indicated an association constant of 5×10^{10} $molar^{-1}$. This binding was

inhibited by sera from patients with Graves' disease who had TSH receptor autoantibodies (detectable by inhibition of labelled TSH binding) (Table 1). ^{125}I-labelled hMAb TSHR1 Fab also bound to TSH receptor coated tubes (association constant by Scatchard analysis=4.5×10^{10} molar^{-1}) and this binding was inhibited by TSH receptor autoantibody positive Graves' sera (Table 2). In addition, detergent solubilised preparations were able to bind to plastic tubes coated with hMAb TSHR1 and this binding could be inhibited by sera containing TSH receptor autoantibodies (Table 3).

[0301] As shown in Table 4 hMAb TSHR1-biotin bound to TSH receptor coated ELISA plates and the binding was inhibited by the international reference preparation NIBSC 90/672 and serum from patients with Graves' disease. Inhibition of binding was not observed by sera from healthy blood donors. FIG. 3a shows a graphical representation of a comparison between an assay for TSHR autoantibodies based on hMAb TSHR1-biotin and earlier assays. The sensitivity of the assay based on hMAb TSHR1-biotin is clearly superior according to concentration of the international standard NIBSC 90/672 detectable. This was confirmed in a study of sera from 72 patients with Graves' disease shown in FIG. 3c. Healthy blood donor sera (n=100) and sera from subjects with non-thyroid diseases (n=43) gave respectively values of up to 10% inhibition of hMAb TSHR1 binding and up to 11% inhibition of TSH binding in this study.

[0302] hMAb TSHR1 IgG did not react with full length TSH receptor preparations on Western blot analysis nor did it react well with ^{35}S-labelled full length TSH receptor in the immunoprecipitation assay nor in the TSH receptor peptide ELISA. This lack of reactivity indicates that hMAb TSHR1 reacts with conformational rather than linear epitopes on the TSH receptor.

[0303] Sequence analysis of the genes coding for hMAb TSHR1 indicated that the heavy chain V region genes were of the VH5 family, the D gene of the D6-13 family and the J gene of the JH5 family and for the light chain the V-gene region is from the VL1-11 germline and the J-gene region is from the JL3b germline. The heavy chain nucleotide and amino acid sequences are shown in FIGS. 4 and 5 respectively and the light chain nucleotide and amino acid sequences are shown in FIGS. 6 and 7 respectively. These sequences are a refinement of the HC and LC nucleotide sequences determined using PCR primers which are degenerate. In particular a HC sequencing artefact for nucleotides 115-120 was identified. Sequencing indicated cagctg (transcribed to amino acids His Val) whereas the crystal structure more reliably indicated amino acids Gln Leu (corresponding bases bcng cagctg). Crystal structure analysis also enabled refinement of the HC and LC derived amino acid sequences particularly in the degenerate PCR primer region. In the case of the LC amino acid 2 was found to be Pro by RT-PCR but was Thr from the crystal structure. In the case of the HC amino acid 2 was found to be Met by RT-PCR but was Val from the crystal structure.

[0304] Comparison of the activities of hMAb TSHR1 IgG preparations and the international standard for TSH receptor autoantibodies in terms of inhibition of labelled TSH binding are shown in Table 5. This enabled a specific activity of hMAb TSHR1 IgG to be estimated as 138 units of NIBSC 90/672 per mg of protein when the assays were carried out in serum and 163 units per mg when the assays were carried out in assay buffer (mean of the 2 values=150 units/mg). hMAb TSHR1 Fab preparations were 288 and 309 units per mg in serum and assay buffer respectively (mean of the 2 values=300 units/mg). Table 6 shows a similar analysis of the lymphocyte donor serum and the donor serum IgG. As can be seen the donor serum contains a mean of 0.38 units/mL of NIBSC 90/672 (0.36 and 0.4 in serum and assay buffer respectively) and the donor serum IgG has a mean specific activity of 0.059 units per mg of protein. These results are summarised in Table 7 and comparison with the specific activity of hMAb TSHR1 IgG (150 units/mg) indicates that the monoclonal autoantibody IgG is 2500 times more active than the lymphocyte donor serum IgG in terms of inhibition of TSH binding.

[0305] Initial assessment of the activities of the various IgG and serum preparations in terms of stimulation of cyclic AMP in CHO cells transfected with the TSH receptor are also shown in Table 7. The stimulation of cyclic AMP assay is characterized by considerable within assay and between assay variability. This relates to several factors including variation in the number and quality of cells initially seeded into the 96 well plates and variation in growth rate of the seeded cells over the subsequent 48 hours. Consequently the assays of hMAb TSHR1 IgG and Fab, lymphocyte donor serum and serum IgG and NIBSC 90/672 were carried out repeatedly and the results are summarized in Table 8. The specific activity of the hMAb TSHR1 IgG was 318 units per mg in the stimulation assay compared with 0.1 units per mg for the lymphocyte donor serum IgG ie the monoclonal autoantibody IgG was about 3000 times as active as the donor serum IgG in terms of stimulation of cyclic AMP production. This value is in reasonable agreement with the value of 2500 times observed for inhibition of TSH binding measurements (see above and Tables 5 and 6). Table 9 shows a further analysis of the TSH receptor stimulating effects of hMAb TSHR1 IgG and Fab and the lymphocyte donor serum IgG.

[0306] The effects of porcine TSH and hMAb TSHR1 IgG on stimulation of cyclic AMP production in CHO cells expressing the TSH receptor were additive as can be seen in the results shown in Table 10.

[0307] Typical results observed in the stimulation of cyclic AMP assay with the reference preparation NIBSC 90/672 are shown in Table 11.

[0308] Tables 12 and 13 show the effects of the various *E. coli* culture supernatants in terms of inhibition of labelled TSH binding and stimulation of cyclic AMP production respectively. Transformed (with hMAb THSR1 plasmid) and IPTG induced cultures of both strains of *E. coli* produced sufficient amounts of recombinant hMAb TSHR Fab to act as potent inhibitors of TSH binding (Table 12) and powerful stimulators of cyclic AMP production (Table 13). Control culture supernatants (from untransformed cells and transformed but non-induced cells) did not produce detectable levels of binding inhibition (Table 12) or stimulating (Table 13) activities.

[0309] In further control experiments, a recombinant human antibody Fab produced by cloning and expression of the HC and LC of a human monoclonal antibody to GAD (4B4) were analysed. Assays of culture supernatants and